Heterogeneous addiction to transforming growth factor-beta signalling in recessive dystrophic epidermolysis bullosa-associated cutaneous squamous cell carcinoma

Dayal, J. H. S.; Mason, S. M.; Salas-Alanis, J. C.; McGrath, J. A.; Taylor, R. G.; Mellerio, J. E.

Published in:
British Journal of Dermatology

DOI:
10.1111/bjd.19421

Publication date:
2021

Document Version
Peer reviewed version

DR JOHN MCGRATH (Orcid ID : 0000-0002-3708-9964)

DR JEMMA MELLERIO (Orcid ID : 0000-0002-2670-8117)

PROFESSOR GARETH INMAN (Orcid ID : 0000-0002-6264-4253)

Article type : Original Article

Heterogeneous addiction to TGFβ signalling in recessive dystrophic epidermolysis bullosa associated cutaneous squamous cell carcinoma

J.H.S. Dayal,[1,2] S.M. Mason,[1] J.C. Salas-Alanis,[3] J.A. McGrath,[4] R.G. Taylor,[2] J.E. Mellerio,[4] K. Blyth,[1,5] A.P. South[6] and G.J. Inman[1,2,5]

1. Cancer Research UK Beatson Institute, Glasgow, UK.
2. Division of Cancer Research, Ninewells Hospital and Medical School, Jacqui Wood Cancer Centre, University of Dundee, UK.
3. Department of Basic Sciences, Health Sciences Division, Universidad de Monterrey, Guadalupe, Nuevo León, México.
4. St. John's Institute of Dermatology, King's College London (Guy's Campus), UK.
5. Institute of Cancer Sciences, University of Glasgow, Glasgow, UK.
6. Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA.

Corresponding author: Professor Gareth J Inman,
Email: Gareth.Inman@glasgow.ac.uk

Funding: This work was supported by Debra UK project grants to GJI and APS and Cancer Research UK core funding, A17196 (KB, GJI).

Conflict of Interest: The authors declare that no conflicts of interest exist.

Running head: TGFβ signalling as a therapeutic target for RDEB cSCC

What's already known about this topic?

- Canonical transforming growth factor beta (TGFβ) signalling is active in RDEB patients' skin and cSCC tumours but its role in tumourigenesis is unknown.
- There is no evidence of mutational inactivation of canonical TGFβ signalling in RDEB cSCC.

What does this study add?

- Exogenous TGFβ stimulation of patient derived RDEB cSCC tumour cells (PDTCs) activates canonical signalling and inhibits cell proliferation in all samples *in vitro*.
- Inhibition of endogenous TGFβ signalling inhibits proliferation, clonogenicity, migration and invasion in the majority of PDTCs but can also have no effect or promote proliferation and clonogenicity in some PDCTs.

What is the translational message?

- Endogenous TGFβ signalling has potential tumour promoting effects in the majority of RDEB cSCCs but may also have tumour supressing effects in some samples.
- TGFβ signalling is an attractive potential therapeutic target in many but not all RDEB cSCCs.
- Biomarkers for TGFβ addiction need to be developed for patient stratification.

Summary

Background: Recessive dystrophic epidermolysis bullosa (RDEB) is associated with a high mortality rate due to the development of life threatening, metastatic cutaneous squamous cell carcinoma (cSCC). Elevated transforming growth factor β (TGFβ) signalling is implicated in cSCC development and progression in RDEB patients.

Objective: To determine the effect of exogenous and endogenous TGFβ signalling in RDEB cSCC with a view to assess the potential of targeting TGFβ signalling for RDEB cSCC therapy.

Methods: A panel of 11 patient derived RDEB cSCC primary tumour keratinocyte cell lines (SCCRDEBs) were tested for their signalling and proliferation responses to exogenous TGFβ. Their responses to TGFBR1 kinase inhibitors (SB-431542 and AZ12601011(AZA01)) was tested using *in vitro* proliferation, clonogenicity, migration, 3D invasion assays and *in vivo* tumour xenograft assays.

Results: All SCCRDEBs respond to exogenous TGFβ by activation of canonical SMAD signalling and proliferative arrest. Blocking endogenous signalling by treatment with SB-431542 and AZ12601011 significantly inhibited proliferation (n=7/11), clonogenicity (n=6/11), migration (n=8/11) and invasion (6/11) of SCCRDEBs. However, these TGFBR1 kinase inhibitors also promoted proliferation and clonogenicity in 2/11 SCCRDEBs. Pre-treatment of *in vitro* TGFBR1 addicted SCCRDEB70 cells with SB-431542 enhanced overall survival and reduced tumour volume in subcutaneous xenografts but had no effect on non-addicted SCCRDEB2 cells in these assays.

Conclusion: Targeting TGFBR1 kinase activity may have therapeutic benefit in the majority of RDEB cSCCs, however, the potential tumour suppressive role of TGFβ signalling in a subset of RDEB cSCCs necessitates biomarker identification to enable patient stratification before clinical intervention.

Introduction

Epidermolysis bullosa (EB) is a skin fragility disorder caused by mutations in genes essential for maintaining the structure and function of the skin. EB has been classified into ~30 clinical subtypes [1] including recessive dystrophic epidermolysis bullosa (RDEB). RDEB is characterised by mutations in the gene encoding type VII collagen, *COL7A1* [2,3], which is the main component of anchoring fibrils that anchors the dermis to

the epidermis. Mutations in *COL7A1* result in either absent or non-functional anchoring fibrils causing acute skin blistering and chronic wounding in RDEB patients [4]. Symptom severity in these patients often results in sepsis and renal failure [5], however, the most significant complication in RDEB patients is the onset of aggressive and potentially metastatic cutaneous squamous cell carcinomas (cSCC) resulting in a high mortality rate, with a cumulative risk of ~90% by age 55 [6]. The exact mechanisms underlying tumour development and rapid progression from primary to metastatic cSCC in RDEB patients is unknown.

Transforming growth factor β (TGFβ) signalling is elevated in RDEB and RDEB cSCC derived primary cells and patient tissue[7-9]. TGFβ is a pleiotropic cytokine that signals through a heterotetrameric complex of its receptors TGFBR1 and TGFBR2. Canonical TGFβ signalling involves receptor-mediated activation of SMAD transcription factors (SMAD2 and SMAD3) that can influence the expression of hundreds of target genes. TGFβ can suppress tumourigenesis in epithelial cells by inducing growth arrest [10]. In contrast, TGFβ can regulate pro-tumourigenic processes such as induction of epithelial to mesenchymal transition (EMT) and promotion of migration, invasion and metastasis[11-13]. It is unclear whether TGFβ functions as a tumour suppressor or promoter in RDEB cSCC disease pathogenesis. Treatment with the angiotensin II type 1 receptor blocker, Losartan[14], or lentiviral mediated delivery of Decorin[15] alleviates RDEB associated fibrosis and mitten deformities in COL7A1-hypomorphic mice. The contribution of inhibition of TGFβ signalling mediated by these treatments to disease inhibition is unclear and the effects of inhibiting TGFβ signalling on chemically induced carcinogenesis in this model[16] is yet to be explored. Recent studies from our lab indicate that TGFβ acts as a potent tumour suppressor in sporadic cSCC where ~30% of cSCCs harbour either *TGFBR1* or *TGFBR2* mutations, leading to the inactivation of canonical signalling [17], and mutational alteration in TGFβ signalling [18] and reduction in canonical signalling activity correlates with markers of disease progression [17-19]. In contrast, mutations in *TGFBR1* or *TGFBR2* have not been found in RDEB cSCC [20], indicative of potential tumour promoting canonical TGFβ signalling in these patients. Here, we use a panel of RDEB cSCC patient derived keratinocytes (SCCRDEBs) and test their response to exogenous TGFβ stimulation and inhibition of endogenous TGFβ signalling in a range of *in vitro* assays and

in vivo subcutaneous xenografts to shed further light on the therapeutic potential of TGFβ signalling inhibitors in RDEB cSCC.

Methods

All patient samples were acquired following informed written consent according to the Helsinki guidelines.

Primary keratinocyte culture

Primary keratinocytes from normal, RDEB and RDEB cSCC patients were isolated, validated by STR profiling in house and cultured as previously described[21-23]. *COL7A1* mutations were validated in early passage RDEB and RDEB cSCC primary cells as previously published[20,24]. Details of the RDEB cSCC cells used in this study are provided in Supplementary Table 1.

Proliferation assays

Cells were treated with either 5ng/ml TGFβ1 (100-21, PeproTech EC Ltd, London,UK) or 0.1% BSA/4mM HCL (carrier), or with 10μM TGFBR1 SB-431542[25] (1614, Tocris, UK) and/or AZA01 (AZ12601011, AstraZeneca)[26] or 0.5% DMSO (D2650, Sigma-Aldrich, UK) as a vehicle control. Proliferation was assessed using the CellTox™ Green cytotoxicity assay (G8731, Promega, UK) as previously described[22]. Cells were imaged for 8 to 10 days using the IncuCyte Zoom® live cell imaging system (Essen bioscience). Data were analysed using the IncuCyte Zoom® inbuilt software.

siRNA mediated depletion of TGFBR1

TGFBR1 knock down was achieved using two individual on-target plus siRNAs (J-003929-09, J-003929-10, Dharmacon-Horizon discovery, UK) and compared to the on-target plus non-targeting siRNA #1 control (D-001810-01, Dharmacon-Horizon discovery, UK). Cells were transfected using lipofectamine® RNAiMAX (13778100, ThermoFisher Scientific, UK) according to the manufacturer's protocol and incubated for 48 hrs prior to seeding in downstream assays.

Clonogenicity assay

The clonogenic potential of primary cells was assessed as previously described[27,28]. Briefly, 3 or 5 cells per well were seeded in a 96 well plate and treated with 10μM SB-431542/AZA01 or 0.5% DMSO the next day. Cells were incubated for 2 weeks to allow colonies to form and stained with 0.4%(w/v) sulforhodamine B (SRB)/1%(v/v) acetic acid solution. Colonies containing more than 50 cells as assessed microscopically were counted and surviving fractions calculated as previously described[27,28].

Wound heal assays

SCCRDEBs were seeded in a 96 well ImageLock plate (4379, Essen biosciences, UK) to achieve a confluent monolayer. The next day, cells were washed and cultured overnight in serum starved conditions (0% FBS) to inhibit proliferation (confirmed by Incucyte tracking, data not shown). Consistent wounds were inflicted using the wound maker (4493, Essen Bioscience, UK), cells were washed in PBS and then incubated in serum free medium with either 10μM SB-431542 or 0.5% DMSO and imaged every 2 hrs using the IncuCyte Zoom® until wound closure. Relative wound density was calculated using the in-built software.

Immunoblotting

Protein lysates were prepared in 4X SDS sample buffer and analysed using SDS-PAGE and western blotting using the following antibodies: PO_4-SMAD2 (Ser465/467) (3101, Cell Signaling Technology, USA), PO_4–SMAD3 (Ser 423/425) (ab52903, Abcam, UK), SMAD2/3 (610842, BD Transduction Laboratories-Europe), SMAD4 (B-8, Santa Cruz Biotechnology, UK) and TGFBR1 (V-22, Santa Cruz Biotechnology, UK). β-actin (A2228, Sigma-Aldrich, UK) or GAPDH (G8795, Sigma-Aldrich, UK) were used as the loading controls.

Immunohistochemistry

4μM thick cryosections or formalin fixed paraffin embedded tissue sections were stained and imaged as previously described using our published immunohistochemistry (IHC) protocol [19] for detecting PO_4-SMAD3 (ab52903, Abcam, UK).

3D organotypic invasion assays

SCCRDEBs were seeded on a dermal equivalent Matrigel (734-1100, Corning®, VWR) – type 1 collagen (C3867, Sigma-Aldrich, UK) 1:1 gels using a previously published protocol [29]. Matrigel-collagen1 gels containing fibroblasts were prepared along with either 10μM SB-431542 or 0.5% DMSO. SCCRDEBs were seeded on top of the gels (with either SB-431542 or DMSO) and incubated overnight. Gels were lifted to air-liquid interface and incubated with SB-431542 or DMSO for 7 days before harvesting. Invasion indexes were calculated as previously described[30].

Subcutaneous Xenografts

All xenograft work was carried out in the Cancer Research UK Beatson Institute with ethical approval from University of Glasgow under the revised Animal (Scientific Procedures) Act 1986 and the EU Directive 2010/63/EU (PPL 70/8645). 8 week old female SCID mice (Charles River, UK) were injected subcutaneously with the indicated number of SCCRDEBs transduced with the lentiviral based imaging vector, BLIV 2.0 Reporter: CMV-Luciferase-EF1a-copGFP-T2A-Puro (BLIV513PA-1, Systems biosciences, CA, USA). In drug treatment experiments, cells were pre-treated with either 10μM SB-431542 or DMSO for four days prior to injection. 5 mice for SCCRDEB2 and 10 mice for SCCRDEB70 were used per treatment group. Mice were monitored three times weekly by calliper measurements and humanely sacrificed when tumours reached the clinical endpoint of ulceration. Tumour volumes were calculated using the formula $(LxW^2)/2$ where L= length and W= width.

Statistical analysis

An ordinary two-way analysis of variance (ANOVA) was conducted of end-point cell-count assays of TGFβ1, SB-431542, SB-505124 and AZA01 against the appropriate vehicle controls. A two-way repeat measures analysis of variance was run on wound heal assays to determine the effect of SB-431542 versus the vehicle control on cell migration across multiple time points. Three separate experiments of 3D invasion and clonogenicity assays were assessed for significance using the student's t.test with Bonferroni correction. Tumour end-point survival was assessed using Kaplan-Meier analysis. Adjusted p-values are presented with values considered significant when $p < 0.05$. Analysis and graphing were conducted using GraphPad Prism or SPSS.

Results

TGFβ signalling is active in epidermal skin and primary keratinocytes

We recently reported that TGFβ canonical signalling as measured by PO_4-SMAD3 expression was primarily localised to the hair follicle stem cells in normal skin, is active in peri-lesional and cSCC tissue and decreased with markers of disease progression[17-19]. We employed the same method using tissue sections from six RDEB cSCC patients and detected nuclear localisation of PO_4 –SMAD3 in all RDEB cSCC samples (representative samples shown in Figure 1a). PO_4-SMAD3 staining was observed in both the dermal and epidermal components of the skin with staining also present in the perilesional epidermis.

Treatment with exogenous TGFβ1 showed induction of PO_4-SMAD2 and PO_4-SMAD3 in primary keratinocytes isolated from the skin of one normal patient (NHKBr1), one RDEB (non-cSCC) patient (RDEB84K) and eleven patient derived SCCRDEBs (SCCRDEB2, 3, 4, 53, 62, 70, 71, 99, 106, 108 and 121). These findings confirm that canonical TGFβ signalling is intact in all of these epidermal keratinocyte populations (Figure 1b, Figure S1). An endogenous level of PO_4-SMAD2/3 was also detected suggestive of autocrine TGFβ signalling, which was reduced by treatment with the TGFBR1 kinase inhibitor, SB-431542.

TGFβ1 can induce growth arrest in epithelial cells during early carcinogenesis [31]. In line with these previous findings, proliferation of primary keratinocytes isolated from normal and non-cSCC RDEB patient skin was significantly inhibited upon treatment with exogenous TGFβ1. All SCCRDEBs (n=11/11) were also significantly inhibited in a proliferation assay following the addition of exogenous TGFβ1 compared to the vehicle control (Figure 2, Figure S2a). Contrary to SCCRDEBs, primary keratinocytes isolated from a subset of sporadic cSCCs do not respond to TGFβ1 in a proliferation assay as ~30% of sporadic cSCCs harbour mutations in *TGFBR1* and *TGFBR2* genes [17], rendering the signalling pathway inactive. Here we show that unlike many sporadic cSCCs, all SCCRDEBs maintain an active TGFβ canonical signalling pathway during cSCC tumourigenesis and can respond to exogenous cytokine by proliferative arrest (summarised in Table 1).

SCCRDEBs exhibit heterogeneous proliferation responses to treatment with TGFBR1 kinase inhibitors

Inhibition of endogenous TGFβ signalling using SB-431542 resulted in the promotion of proliferation in NHKs and RDEBKs (Figure 3), consistent with the growth inhibition observed following treatments with exogenous TGFβ1. However, interestingly, proliferation of 63% (n=7/11) of SCCRDEBs (SCCRDEB4, 62, 70, 71, 99, 106 and 108) was significantly inhibited upon treatment with SB-431542 (Figure 3, Figure S2a). Blocking endogenous signalling significantly promoted proliferation of SCCRDEB2 and SCCRDEB121, whereas, SCCRDEB3 and SCCRDEB53 did not respond to SB-431542 in this assay (Figure 3, Figure S2a). These results were validated using two additional TGFBR1 kinase inhibitors AZA01 and SB-505124, in a promoted (SCCRDEB2) and an inhibited (SCCRDEB70) cell line in a proliferation assay and kinase inhibition was confirmed using immunoblotting as indicated by loss of expression of PO_4-SMAD2 (Figure S2b-c). These data confirm that SCCRDEBs show an inherent heterogeneity in response to blocking endogenous TGFβ signalling in a proliferation assay.

SCCRDEBs exhibit heterogeneous clonogenic potential following treatment with TGFBR1 kinase inhibitors

The clonogenic potential of cells is reflective of their stem cell like properties and can be measured by assessing the colony forming ability of cancer cells in low-density seeding conditions[32]. SCCRDEBs were seeded at low cell densities and treated with SB-431542 and AZA01 to assess the effects of blocking TGFβ signalling on colony formation. The surviving fraction was calculated following treatment with the inhibitor as previously described [27]. Six out of 7 of the SCCRDEBs that were inhibited in the proliferation assay were also significantly inhibited in the clonogenicity assay following treatment with TGFBR1 kinase inhibitors (Figure 4A, Figure S3, Table 2). SCCRDEB71 cells did not form colonies. SCCRDEB2 and SCCRDEB121 that were promoted in the proliferation assay also showed an enhanced clonogenic potential upon addition of TGFBR1 kinase inhibitors (Figure 4a, Table 2); thereby confirming the heterogeneity observed in response to inhibiting endogenous TGFβ signalling in SCCRDEBs.

To confirm the on-target effects of the kinase inhibitors, two individual siRNAs were used to deplete the expression levels of TGFBR1 and the clonogenic potential of SCCRDEB2,4 and 62 cells was tested following knock down of *TGFBR1*. Knockdown efficiency was assessed by western blotting (Figure 4b). Clonogenicity of SCCRDEB4 and SCCRDEB62 cells was significantly reduced following knock down using both siRNAs targeting *TGFBR1* when compared to the non-targeting control (Figure 4b). Efficient knockdown of TGFBR1 (with TGFBR1-si10) promoted clonogenicity of SCCRDEB2 cells (Figure 4b). These data confirmed that the effect of the TGFBR1 kinase inhibitors in these cells is on-target.

SB-431542 inhibits the migration and invasion of TGFBR1 addicted SCCRDEBs

So far our data indicates that two thirds of the SCCRDEBs are addicted to the endogenous kinase activity of TGFBR1 for efficient proliferation and clonogenicity. Epithelial cells undergo morphological changes to acquire a pro-migratory and invasive phenotype during carcinogenesis and TGFβ can drive migration and invasion of cancer cells in a context dependent and tumour cell specific manner[10,12,13,33]. Consistent with this, exogenous TGFβ1 treatment enhanced migration of SCCRDEB2, 4, 71, 99 and 106 cells, inhibited migration of SCCRDEB53 cells and no effect on migration of SCCRDEB3, 62, 70, 108 and 121 cells in a 2D wound heal scratch assay (Figure S4, Table 1). Treatment with SB-431542 significantly impaired cell migration of SCCRDEB4, 62, 70, 99,108 and 121 cells (Figure 5a, Figure S5, Table 2) suggesting a role of endogenous TGFβ signalling in promoting a pro-migratory phenotype in these cells. SCCRDEB2, 3 and 53 cells did not respond to TGFBR1 kinase inhibitors in this assay (Figure S5, Table 2), providing further evidence of heterogeneity of SCCRDEB responses to TGFBR1 kinase inhibitors.

Matrigel-type1 collagen gels were used to develop 3D skin equivalents to test the invasive potential of SCCRDEBs in the presence of fibroblasts derived from RDEB cSCC patients. Invasion assays were carried out either in the presence or absence of SB-431542 to block endogenous TGFβ signalling and the invasion index was calculated as previously described [30]. The invasive potential of SCCRDEB4, 62, 70, 99, 106 and 108 cells was inhibited by SB-431542 (Figure 5b, Figure S6, Table 2), mimicking the effects

of the kinase inhibitor in the proliferation, clonogenicity and 2D wound heal assays in the same cells. SB-431542 did not inhibit invasion of SCCRDEB71 cells (Figure S6), despite inhibiting proliferation and migration of these cells (Table 2). SCCRDEB2 and SCCRDEB121 cells which were promoted in proliferation and clonogenicity assays by SB-431542 did not show any significant response to SB-431542 in the 3D organotypic invasion assay (Figure 5b, Table 2). These data confirm that SCCRDEB2 and SCCRDEB121 cells are inherently different to SCCRDEB4/62/70/99/106/108 cells in their response to inhibition of TGFBR1 endogenous signalling and that TGFBR1 activity in SCCRDEB2, SCCRDEB71 and SCCRDEB121 cells may not play a role in driving invasion in these cells.

Targeting TGFBR1 inhibits *in vivo* tumour growth of SCCRDEB70 but not SCCRDEB2 cells.

To determine *in vivo* tumourigenicity we performed pilot serial dilution subcutaneous xenograft experiments using SCCRDEB70, 62, 4 and 2 cells. Consistent tumour growth was observed with $4x10^4$-$4x10^6$ cells depending on the cell line (Figure S7). H&E staining of tumours harvested at the endpoint showed the presence of tumour cells and ulceration (Figure S7). SCCRDEB4 cells did not form tumours in this assay. Consistent with our *in vitro* analysis, animals transplanted with SB-431542 pre-treated "TGFBR1 addicted" SCCRDEB70 cells showed enhanced survival in *in vivo* subcutaneous xenografts reaching clinical endpoint later when compared to the vehicle control (DMSO) pre-treated cells (Figure 6a). Tumour initiation was not affected as 9 out of 10 mice formed tumours in the DMSO treated group, whereas, all 10 mice formed tumours in the SB-431542 treated group. At week 5 tumour volumes of SB-431542 pre-treated xenografts was significantly reduced compared to the vehicle control xenografts (Figure 6b). Mice injected with SB-431542 pre-treated SCCRDEB2 cells that were promoted in *in vitro* proliferation and clonogenicity assays by this treatment, tended to reach clinical endpoint earlier compared to mice injected with DMSO pre-treated cells (Figure S8a), however this finding did not reach statistical significance. No significant difference was observed in tumour volumes between control or pre-treatment groups (Figure S8b). Overall, our *in vivo* experiments are consistent with our *in vitro* studies indicating that inhibition of TGFβ signalling in some SCCRDEBs will restrict proliferation and tumour growth.

Discussion

Management of life threatening cSCC arising in RDEB patients is challenging with no approved treatment options and limb amputation often used as the last resort to combat disease progression [34]. Radiotherapy and chemotherapy are available as palliative care options with little evidence to show any significant impact on overall survival [34]. Recent work has identified PLK1 as a potential target [35] and ongoing phase II clinical trials with rigosertib may provide a much needed opportunity for therapeutic targeting of RDEB cSCC.

Elevated TGFβ signalling has been reported in RDEB skin and postulated as the underlying cause for driving inflammation, fibrosis and extracellular matrix remodelling and thereby disease severity in RDEB patients [7,14-16]. TGFβ has been shown to act as both a tumour promoter and suppressor in carcinogenesis in a context dependent manner [36]. It is unknown whether TGFβ signals as a tumour suppressor or a tumour promotor in cSCC development in RDEB patients and therefore if it is an attractive therapeutic target for cSCC prevention or treatment. Here we also found TGFβ signalling is active in RDEB cSCC tissue and primary keratinocytes confirming previous findings (Figure 1a, 1b, S1). Following the validation of intact active canonical TGFβ signalling we addressed the question of the potential use of targeting TGFβ signalling for therapeutic use in RDEB cSCC patients. We found that exogenous TGFβ treatment inhibited cell proliferation in all of the RDEB cSCC cells indicative of a potential tumour suppressor role (Summarised in Table1). Maintenance of canonical signalling and potentially tumour suppressive anti-proliferative activity in all RDEB samples studies is in stark contrast to our findings in sporadic cSCC, where, mutational inactivation of canonical TGFβ signalling occurs in ~30% of tumours which results in loss of anti-proliferative tumour suppressive effects[17]. This finding indicates that loss of tumour suppressive TGFβ signalling is not required for cSCC development in RDEB patients and that TGFβ signalling may act as a tumour promoter.

Despite the universal anti-proliferative response of SCCRDEBs to exogenous TGFβ we surprisingly observed heterogeneous responses to blockade of endogenous TGFβ signalling using TGFBR1 kinase inhibitors or TGFBR1 siRNA knockdown

approaches in a range of *in vitro* assays (summarised in Table 2), which were validated *in vivo* (Figure 6). TGFβ signalling can act as a potential tumour promoter in approximately two thirds of patient derived SCCRDEBs as inhibition of endogenous signalling blocks proliferation, migration and invasion *in vitro* (Table 2) and tumour growth *in vivo*, suggesting that targeting this pathway may be of significant therapeutic value to patients harbouring these tumours. Importantly, however, we also found that inhibiting endogenous signalling had no effect in 2/11 cell lines and promoted proliferation and clonogenicity in a further 2 indicating that inhibiting TGFβ signalling in these patients may have no clinical benefit or even promote disease progression.

Immunoblotting of SCCRDEBs show no obvious evidence of difference in basal or induced level of TGFβ canonical signalling in the TGFBR1 addicted vs. non-addicted cells as assessed by PO_4-SMAD2/3 expression (Figure 1b and S1), making it difficult to explain the heterogeneity in TGFBR1 inhibition responses of SCCRDEBs based on autocrine TGFβ signalling. Analysis of the publically available whole exome sequencing dataset [20] for the SCCRDEBs (SCCRDEB4 and 121 data not available) we found SCCRDEBs that are not addicted to TGFBR1 activity (SCCRDEB2, 3 and 53) harbour mutations in *FAT1,* whereas, TGFBR1 'addicted' lines are *FAT1* wild type (SCCRDEB 62, 70,71,99,106 and 108) (Figure S9). Somatic mutations in *FAT1* are frequently reported in cancer [37,38] including sporadic cSCC [18]. Functional FAT1 can suppress Wnt/β-catenin signalling, in turn inhibiting proliferation of cancer cells by blocking cell cycle progression at the G1-S checkpoint [38]. Loss of function mutation in *FAT1* is associated with cancer progression and is shown to result in drug resistance via downregulation of the Hippo signalling pathway in breast cancer [39]. Whether treatment with SB-431542 in *FAT1* wild type SCCRDEBs results in dysregulation of the hippo and or Wnt/β-catenin signalling still remains to be tested. Additionally, the role of non-canonical signalling pathways (PI3K/AKT, MAPK etc) and other members of the TGFβ superfamily (including Activins and BMPs, their receptors and downstream SMADs) in determining heterogeneous TGFBR1 addiction needs to be explored to achieve a better understanding of the molecular mechanisms underlying the heterogeneous response to TGFβ signalling inhibition in RDEB cSCC and ensure an informed biomarker driven selective therapeutic intervention in RDEB patients. In the meantime, we countenance

that clinical trials using agents with anti-TGFβ signalling activity in RDEB and RDEB cSCC should proceed with caution due to the potential tumour suppressive function of the canonical TGFβ signalling pathway in some RDEB cSCCs.

Acknowledgements

We would like to thank the Core Services and Advanced Technologies at the Cancer Research UK Beatson Institute (C596/A17196), with particular thanks to the Biological Services Unit and Histology. We would also like to thank Professors Christina Grube and Jon Bauer (Department of Dermatology and EB House Austria, Saltzburg) for supplying RDEB samples.

References

1 Fine JD, Bruckner-Tuderman L, Eady RAJ *et al.* Inherited epidermolysis bullosa: Updated recommendations on diagnosis and classification. *J Am Acad Dermatol* 2014; 70: 1103-26.

2 Christiano AM, Uitto J. Molecular diagnosis of inherited skin diseases: the paradigm of dystrophic epidermolysis bullosa. *Adv Dermatol* 1996; 11: 199-213; discussion 4.

3 Christiano AM, Greenspan DS, Hoffman GG *et al.* A missense mutation in type VII collagen in two affected siblings with recessive dystrophic epidermolysis bullosa. *Nat Genet* 1993; 4: 62-6.

4 Burgeson RE, Lunstrum GP, Rokosova B *et al.* The structure and function of type VII collagen. *Ann N Y Acad Sci* 1990; 580: 32-43.

5 Fine JD, Johnson LB, Weiner M *et al.* Inherited epidermolysis bullosa and the risk of death from renal disease: experience of the National Epidermolysis Bullosa Registry. *Am J Kidney Dis* 2004; 44: 651-60.

6 Fine JD, Johnson LB, Weiner M *et al.* Epidermolysis bullosa and the risk of life-threatening cancers: The National EB Registry experience, 1986-2006. *J Am Acad Dermatol* 2009; 60: 203-11.

7 Ng YZ, Pourreyron C, Salas-Alanis JC *et al.* Fibroblast-derived dermal matrix drives development of aggressive cutaneous squamous cell carcinoma in patients with recessive dystrophic epidermolysis bullosa. *Cancer Res* 2012; 72: 3522-34.
8 Knaup J, Gruber C, Krammer B *et al.* TGF beta-signaling in squamous cell carcinoma occurring in recessive dystrophic epidermolysis bullosa. *Anal Cell Pathol* 2011; 34: 339-53.
9 Odorisio T, Di Salvio M, Orecchia A *et al.* Monozygotic twins discordant for recessive dystrophic epidermolysis bullosa phenotype highlight the role of TGF-beta signalling in modifying disease severity. *Hum Mol Genet* 2014; 23: 3907-22.
10 Massague J. TGFbeta in Cancer. *Cell* 2008; 134: 215-30.
11 Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; 85: 314-23.
12 Inman GJ. Switching TGFbeta from a tumor suppressor to a tumor promoter. *Curr Opin Genet Dev* 2011; 21: 93-9.
13 Batlle E, Massague J. Transforming Growth Factor-beta Signaling in Immunity and Cancer. *Immunity* 2019; 50: 924-40.
14 Nystrom A, Thriene K, Mittapalli V *et al.* Losartan ameliorates dystrophic epidermolysis bullosa and uncovers new disease mechanisms. *EMBO Mol Med* 2015; 7: 1211-28.
15 Cianfarani F, De Domenico E, Nystrom A *et al.* Decorin counteracts disease progression in mice with recessive dystrophic epidermolysis bullosa. *Matrix Biol* 2018.
16 Mittapalli VR, Madl J, Loffek S *et al.* Injury-Driven Stiffening of the Dermis Expedites Skin Carcinoma Progression. *Cancer Res* 2016; 76: 940-51.
17 Cammareri P, Rose AM, Vincent DF *et al.* Inactivation of TGFbeta receptors in stem cells drives cutaneous squamous cell carcinoma. *Nat Commun* 2016; 7: 12493.
18 Inman GJ, Wang J, Nagano A *et al.* The genomic landscape of cutaneous SCC reveals drivers and a novel azathioprine associated mutational signature. *Nat Commun* 2018; 9: 3667.

19 Rose AM, Spender LC, Stephen C *et al.* Reduced SMAD2/3 activation independently predicts increased depth of human cutaneous squamous cell carcinoma. *Oncotarget* 2018; 9: 14552-66.

20 Cho RJ, Alexandrov LB, den Breems NY *et al.* APOBEC mutation drives early-onset squamous cell carcinomas in recessive dystrophic epidermolysis bullosa. *Sci Transl Med* 2018; 10.

21 Watt SA, Pourreyron C, Purdie K *et al.* Integrative mRNA profiling comparing cultured primary cells with clinical samples reveals PLK1 and C20orf20 as therapeutic targets in cutaneous squamous cell carcinoma. *Oncogene* 2011; 30: 4666-77.

22 McHugh A, Fernandes K, South AP *et al.* Preclinical comparison of proteasome and ubiquitin E1 enzyme inhibitors in cutaneous squamous cell carcinoma: the identification of mechanisms of differential sensitivity. *Oncotarget* 2018; 9: 20265-81.

23 Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res* 1981; 41: 1657-63.

24 Pourreyron C, Cox G, Mao X *et al.* Patients with recessive dystrophic epidermolysis bullosa develop squamous-cell carcinoma regardless of type VII collagen expression. *J Invest Dermatol* 2007; 127: 2438-44.

25 Inman GJ, Nicolas FJ, Callahan JF *et al.* SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002; 62: 65-74.

26 Spender LC, Ferguson GJ, Hughes GD *et al.* Preclinical Evaluation of AZ12601011 and AZ12799734, Inhibitors of Transforming Growth Factor beta Superfamily Type 1 Receptors. *Mol Pharmacol* 2019; 95: 222-34.

27 Franken NA, Rodermond HM, Stap J *et al.* Clonogenic assay of cells in vitro. *Nat Protoc* 2006; 1: 2315-9.

28 Spender LC, Ferguson GJ, Liu S *et al.* Mutational activation of BRAF confers sensitivity to transforming growth factor beta inhibitors in human cancer cells. *Oncotarget* 2016; 7: 81995-2012.

29 Pourreyron C, Chen M, McGrath JA *et al.* High levels of type VII collagen expression in recessive dystrophic epidermolysis bullosa cutaneous squamous cell carcinoma keratinocytes increases PI3K and MAPK signalling, cell migration and invasion. *Br J Dermatol* 2014; 170: 1256-65.

30 Jenei V, Nystrom ML, Thomas GJ. Measuring invasion in an organotypic model. *Methods Mol Biol* 2011; 769: 223-32.

31 Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001; 29: 117-29.

32 Fedr R, Pernicova Z, Slabakova E *et al.* Automatic cell cloning assay for determining the clonogenic capacity of cancer and cancer stem-like cells. *Cytometry A* 2013; 83: 472-82.

33 Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Curr Opin Cell Biol* 2009; 21: 166-76.

34 Mellerio JE, Robertson SJ, Bernardis C *et al.* Management of cutaneous squamous cell carcinoma in patients with epidermolysis bullosa: best clinical practice guidelines. *Br J Dermatol* 2016; 174: 56-67.

35 Atanasova VS, Pourreyron C, Farshchian M *et al.* Identification of Rigosertib for the Treatment of Recessive Dystrophic Epidermolysis Bullosa-Associated Squamous Cell Carcinoma. *Clin Cancer Res* 2019.

36 Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. *Trends Cell Biol* 2001; 11: S44-51.

37 Katoh M. Function and cancer genomics of FAT family genes (review). *Int J Oncol* 2012; 41: 1913-8.

38 Morris LG, Kaufman AM, Gong Y *et al.* Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. *Nat Genet* 2013; 45: 253-61.

39 Li Z, Razavi P, Li Q *et al.* Loss of the FAT1 Tumor Suppressor Promotes Resistance to CDK4/6 Inhibitors via the Hippo Pathway. *Cancer Cell* 2018; 34: 893-905 e8.

Figure legends

Figure 1: TGFβ signalling is active in RDEB cSCC tissue and cells

a. Representative images of immunohistochemical analysis of RDEB cSCC patient tissue indicates heterogeneous nuclear localisation of PO_4-SMAD3 (red arrows) in both tumour cells and in the dermal (D) and epidermal (E) components of the skin (perilesional skin Patient1). Scale bar = 100μM. **b.** Western blotting analysis indicates that PO_4-SMAD2/3 is induced following treatment with TGFβ1 (5ng/ml, 2 hrs) in patient derived NHK (NHKBr1), RDEBK (RDEB84K) and RDEB cSCCKs (SCCRDEB2,3,4,53,62 and 70) and that this is blocked by co-treatment with the TGFBR1 kinase inhibitor SB-431542.Total SMAD2/3 and SMAD4 is detectable in all primary keratinocytes tested. Beta-Actin is used as a loading control.

Figure 2: Exogenous TGFβ inhibits proliferation of primary normal and tumour derived keratinocytes

Cell proliferation asssays were performed using the IncuCyte ZOOM® live cell imaging system and the CellTox™ Green cytotoxicity assay (n=3). Exogenous TGFβ1 (5ng/ml) inhibits proliferation of NHK (NHKBr1/Br2), RDEBK (RDEB84K) and SCCRDEBs (SCCRDEB2, 3, 4, 53, 62 and 70) compared to the vehicle control. Endpoint two-way ANOVA, error bars= 95% CI, **** = $p<0.0001$.

Figure 3: SB-431542 promotes proliferation in normal keratinocytes and inhibits or promotes proliferation of a subset of SCCRDEBs

Proliferation was assessed using the IncuCyte ZOOM® live cell imaging system and the CellTox™ Green assay. Patient derived normal human keratinocytes (NHKBr1/Br2), RDEBK (RDEB84K) and SCCRDEBs (SCCRDEB2, 3, 4, 53, 62 and 70) were treated with

SB-431542 (10μM) or DMSO (vehicle control). Proliferation of normal keratinocytes and SCCREDB2 cells was promoted whereas proliferation of SCCRDEBs (SCCRDEB4, 62 and 70) was inhibited. End point two-way ANOVA, N=3, error bars= 95% CI, p<0.0001.

Figure 4: TGFBR1 kinase inhibitors inhibit or promote clonogenic potential of a subset of SCCRDEBs

a. SCCRDEBs were seeded (3 cells/well in middle 60 wells of a 96 well plate) and treated with either DMSO, SB-431542 (10μM) or AZA01 (10μM). Clonogenic potential was assessed 2 weeks post treatment following staining with sulforhodamine B. Upper panels show representative images and lower graphs indicate quantification of surviving fractions, N=3 error bars= 95% CI, two tailed student T-test with corrected p-values according to Bonfferoni principles *=p<0.05, **=p<0.01, ***p=<0.001. The clonogenic potential of SCCRDEB4, 62 and 70 was inhibited whereas it was promoted in SCCRDEB2 and 121 following treatments with SB-431542 and AZA01. **b.** Two siRNAs targeting *TGFBR1* (TGFBR1 si9/si10) also enhanced the clonogenic potential in SCCRDEB2 cells and inhibited clonogenicity of SCCRDEB4 and SCCRDEB62 compared to the non-targeting control siRNA. Representative images shown in upper left panels and surviving fraction quantification is shown in the lower graphs. N=3, error bars= 95% CI. Two tailed student T-test with adjusted p-values according to Bonfferoni principles. *p<0.05. **p<0.01, ***p<0.001. Right hand panels show Western blot analysis confirming loss of expression of TGFBR1 following knockdown using both (Si9 and Si10) TGFBRI siRNAs in all cells. Beta-Actin was used as a loading control.

Figure 5: SB-431542 can inhibit the pro-migratory and pro-invasive phenotype of TGFBR1 addicted SCCRDEBs

a. Real time wound heal-scratch InCucyte Zoom® live cell imaging analysis. SB-431542 (10μM) can inhibit migration of SCCRDEB4, 62 and 70 cells compared to the vehicle control (DMSO). Two-way repeat measures ANOVA, N=3 biological replicates, error bars= 95% CI, ****= p<0.001. **b.** The invasive potential of a panel of SCCRDEBs was assessed using Matrigel-Collagen 1 gels (1:1). RDEB cSCC fibroblasts (SCCRDEB71F) were added along with either DMSO or SB-431542 (10μM) to make up the Matrigel-

collagen dermal equivalents. SB-431542 treatment inhibited the invasive potential of SCCRDEB4, 62 and 70 cells and did not effect this in SCCRDEB2 and SCCRDEB121 cells. Representative H and E images are shown in the upper panels with examples of invading keratinocyte colonies marked with black arrows. Lower graphs show quantification of invasion indexes. N=3 independent experiments, mean±SD. Two tailed student t-test **p<0.01 and * p<0.05, Scale bar = 100μM

Figure 6: Pre-treatment of SCCRDEB70 cells with SB-431542 extends survival and delays tumour growth in a xenograft assay

a. Overall survival of mice subcutaneously injected with SB-431542 (n=10) or DMSO (n=9) pre-treated SCCRDEB70 cells as determined by Kaplan-Meier analysis. The curves show an enhanced survival of mice injected with SB-431542 pre-treated SCCRDEB70 cells compared to the DMSO pre-treated cells (p=0.01). **b.** Calliper measured tumour volume at 5 weeks post injection of SB-431542 pre-treated xenografts is significantly lower than the vehicle pre-treated cohort (Mann-Whitney U test).

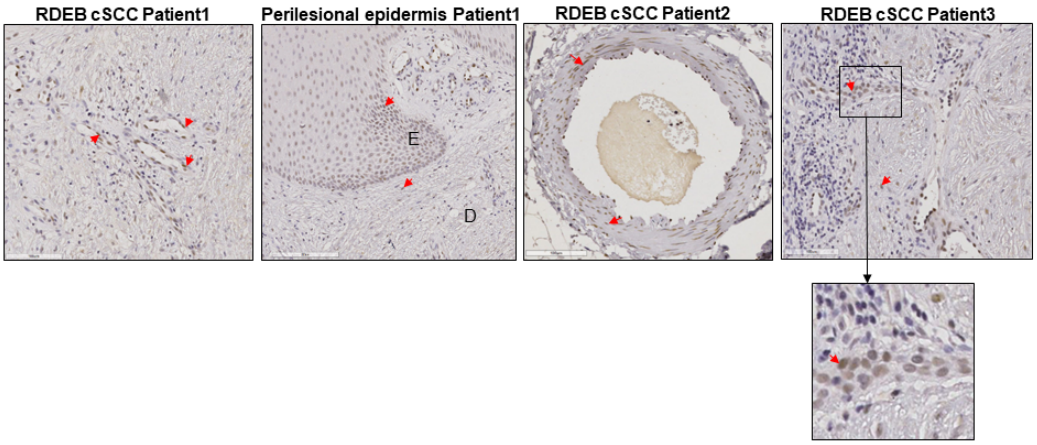
Cells	PO_4-SMAD2	PO_4-SMAD3	Proliferation	2D-Migration
SCCRDEB2	induced	induced	inhibited	promoted
SCCRDEB3	induced	induced	inhibited	no effect
SCCRDEB4	induced	induced	inhibited	promoted
SCCRDEB53	induced	induced	inhibited	inhibited
SCCRDEB62	induced	induced	inhibited	no effect
SCCRDEB70	induced	induced	inhibited	no effect
SCCRDEB71	induced	induced	inhibited	promoted
SCCRDEB99	induced	induced	inhibited	promoted
SCCRDEB106	induced	induced	inhibited	promoted
SCCRDEB108	induced	induced	inhibited	no effect
SCCRDEB121	induced	induced	inhibited	no effect

Table 1: Summary of responses of SCCREDEB cells to exogenous TGFβ1 treatment.

Cells	Proliferation	Clonogenicity	2D-Migration	3D-Invasion
SCCRDEB2	promoted	promoted	no effect	no effect
SCCRDEB3	no effect	no effect	no effect	no effect
SCCRDEB4	inhibited	inhibited	inhibited	inhibited
SCCRDEB53	no effect	no effect	no effect	no effect
SCCRDEB62	inhibited	inhibited	inhibited	inhibited
SCCRDEB70	inhibited	inhibited	inhibited	inhibited
SCCRDEB71	inhibited	no colonies	inhibited	no effect
SCCRDEB99	inhibited	inhibited	inhibited	inhibited
SCCRDEB106	inhibited	inhibited	inhibited	inhibited
SCCRDEB108	inhibited	inhibited	inhibited	inhibited
SCCRDEB121	promoted	promoted	inhibited	no effect

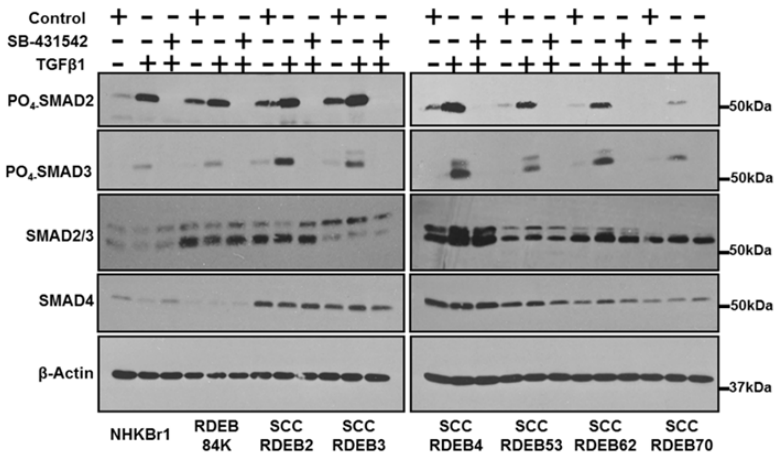
Table 2: Summary of responses of SCCREDEB cells to inhibition of endogenous TGFβ1 signalling.

Figure 1a



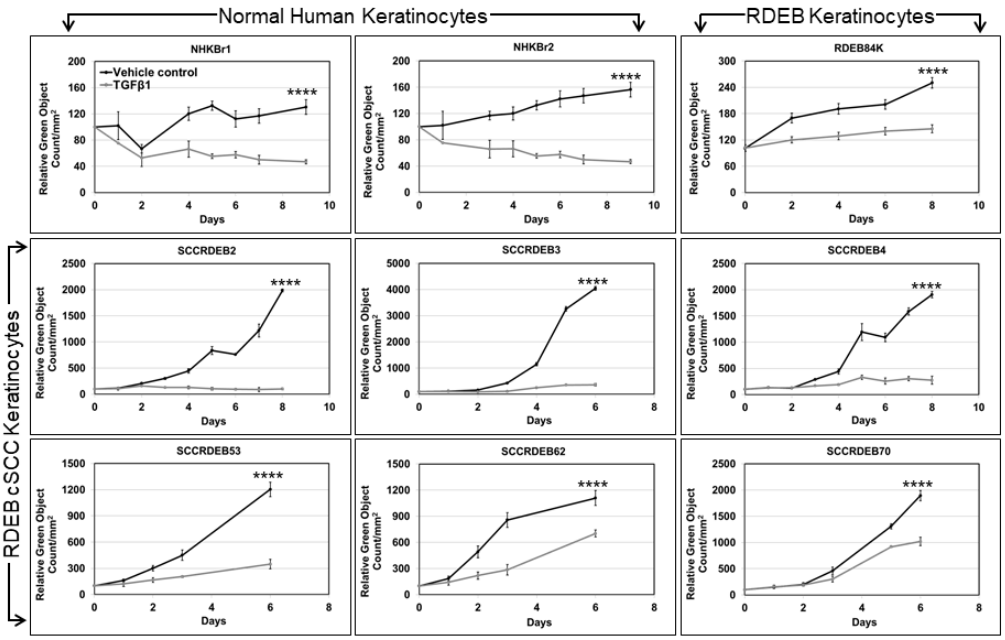
bjd_19421_f1a.tif

Figure 1b



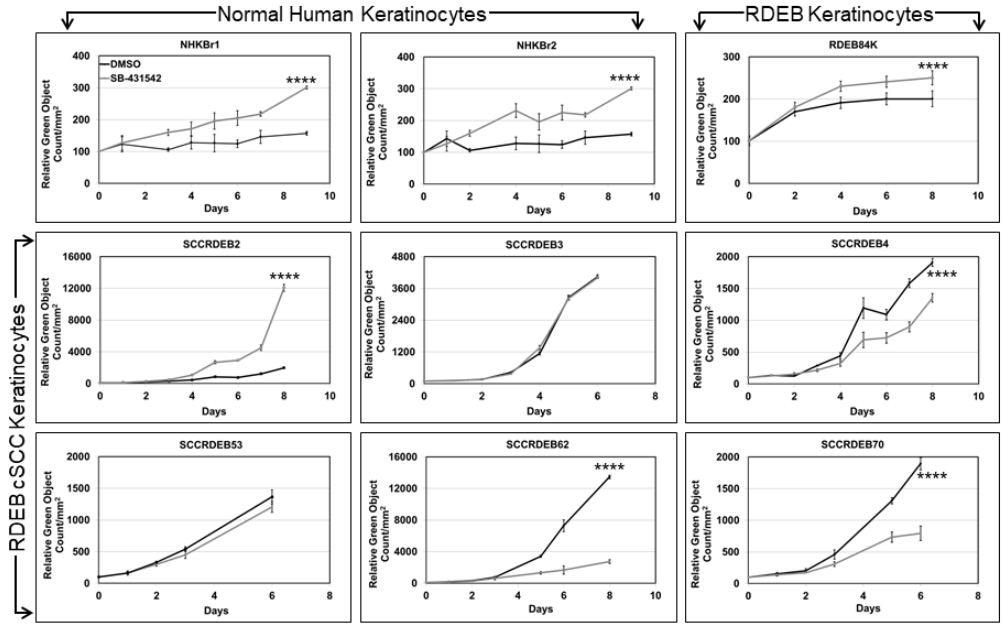
bjd_19421_f1b.tif

Figure 2



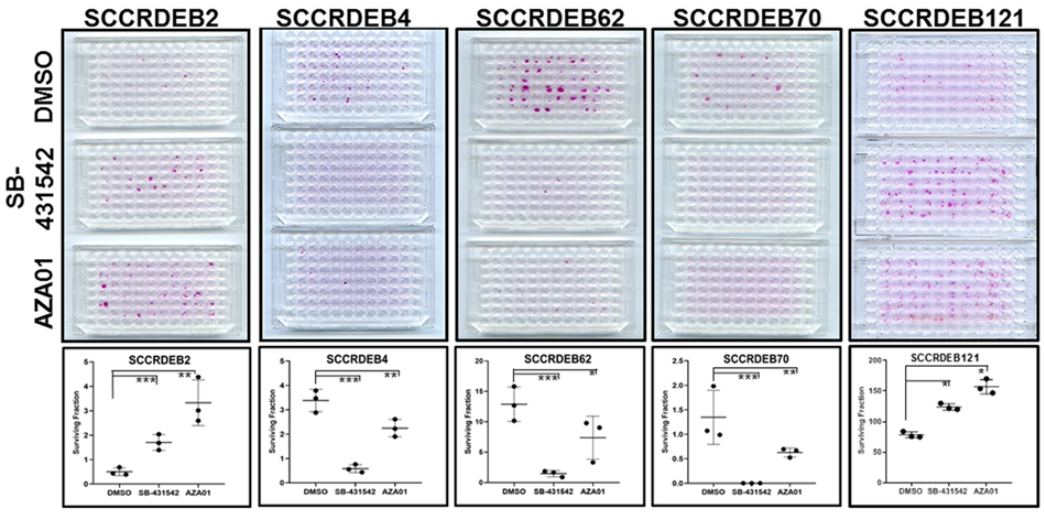
bjd_19421_f2.tif

Figure 3



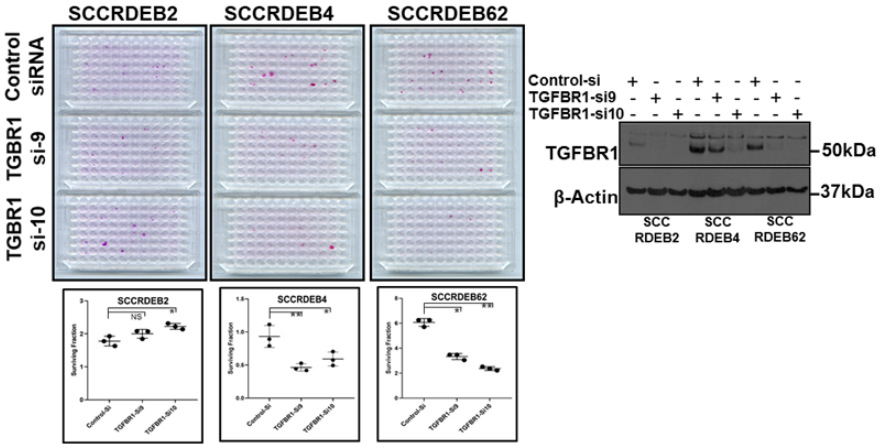
bjd_19421_f3.tif

Figure 4a



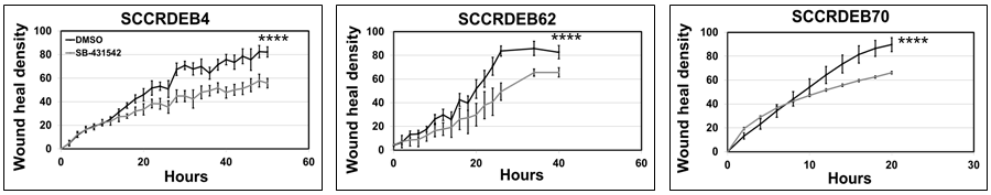
bjd_19421_f4a.tif

Figure 4b



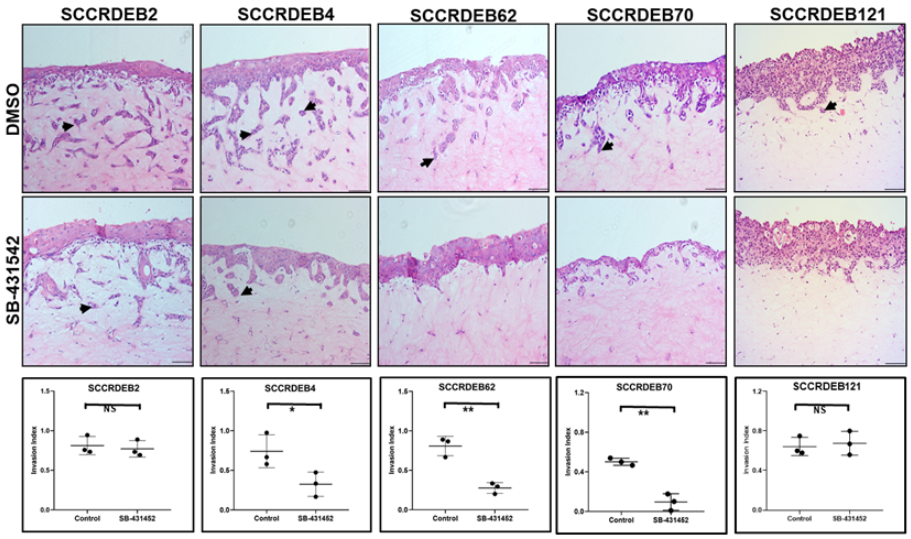
bjd_19421_f4b.tif

Figure 5a



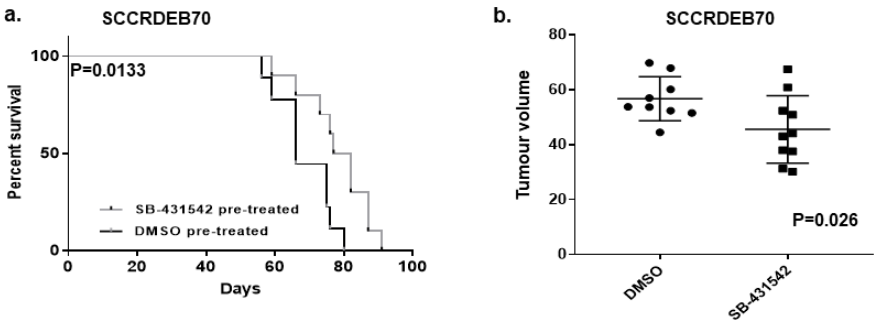
bjd_19421_f5a.tif

Figure 5b



bjd_19421_f5b.tif

Figure 6



bjd_19421_f6.tif